Transformation

1. Thaw chemically competent cell on ice for 10 minutes, for most cases Top10 cells are used.
2. Transform 2-5ul containing about 100ng of plasmid into cells. Carefully flick the tube for 3-5 times to mix cells with plasmid.
3. Incubate on ice for 20 minutes.
4. Heat shock cells for 1min30sec at 42^{o}C
5. Incubate transformed cells on ice for 2 minutes.
6. Add 500ul of LB media into the mixture and let it recovers for 1 hour at 37^{o}C incubator.
7. Warm selection plate during 1 hour incubation.
8. Spread 100ul of cells directly onto antibiotic selection plate. Alternatively, concentrate the cells by centrifuge at 12000rpm for 5 minutes. Remove supernatant and take 100ul of remaining cells to spread.

RT-PCR

1. Inoculate colony containing superoperon plasmid into 5ml LB media with Ampicillin antibiotic added. Let the cell culture grow overnight at 37^{o}C incubator.
2. Harvest the cell at 4700rpm for 7 minutes. Remove supernatant.
3. Resuspend the pellet with 1mL of Trizol/Qiazol. Use immediately or otherwise keep it at -80 ^{o}C.
4. Add 200ul of chloroform. Vortex the mixture briefly for 15 seconds.
5. Incubate the mixture for 3-5 minutes at room temperature.
6. Spin at 12000rpm for 15 minutes at 4 ^{o}C.
7. Transfer the clear supernatant to a new tube. Avoid transferring the pinkish color pellet.
8. Add equal amount of 70% ethanol and mix.
9. Transfer up to 700ul to RNA purification column. Spin at 10000rpm for 30 seconds at 4 ^{o}C.
10. Discard Flow through. Repeat adding the remaining solution into column.
11. Wash with 350ul Buffer RWI.
12. For each column, add 80ul of pre-mixed DNase with Buffer RDD.
13. Incubate at room temperature for 30 minutes. Do not spin until the complete the next step.
14. Add 350ul of Buffer RWI into the column and spin.
15. Wash column with 500ul of Buffer RPE for twice.
16. Then, spin the column for 5 minutes to make sure the column is free from Buffer RPE.
17. Elute with 30-40ul of RNase free water.

Lamba-red mutagenesis

a) Molecular cloning
    1. Set up 10x PCR reactions to amplify FRT-flanked Kanamycin cassette using pKD4 as DNA template.
    2. Combine the 10x PCR reactions together (total 500ul) and aliquot into 2 eppendorf tube with 250ul in each tube.
    3. Add 500ul of 100% ethanol. Mix well.
    4. Incubate on ice for 15min.
    5. Spin at 13000rpm for 5 minutes at 4 ^{o}C.

6. Remove supernatant. Wash the DNA pellet twice with 500ul 70% ethanol. Do not vortex or disturb the DNA pellet.
7. Spin for 1 minute after each wash.
8. Remove supernatant as much as possible.
9. Dry the pellet in Speedyvac for 15 minutes.
10. Resuspend pellets with 25ul Elution Buffer of Miniprep Kit for each tube.

b) Digest concentrated PCR products with DpnI

DpnI digestion is set up as followed:

Concentrated PCR products	50ul
NEB DpnI	1ul
CutSmart buffer	6ul
Milli Q water	3ul
Total reaction volume	60ul

Digest PCR products at 37^{o}C for 2 hours. After this, the PCR products is ready for electroporation.

Electroporation

1. Pre-cool electroporation cuvette and thaw electrocompetent cell on ice.
2. Add 5ul of PCR products into cells. Flick the tube gently to mix.
3. Transfer the mixture to a chilled electroporation cuvette.
4. Electroporate condition is set as voltage: 2.5kV and the time constant is about 3-4 milliseconds.
5. Immediately add 500ul of LB media to the cuvette, gently mix and transfer to the eppendorf tube.
6. Incubate the cells at 37^{o}C incubator for 1 hour.
7. Spread 100ul of cells on pre-warmed antibiotic plate.

Passage mammalian cell

1. Passage cells for 2-3 times per week.
2. Pre-warmed DMEM media, trypsin and PBS buffer.
3. Remove media and wash with 3ml of PBS. Be careful during washing step to avoid detach the cells at this moment.
4. Remove PBS buffer and add 5ml of trypsin.
5. Incubate the cells with trypsin added for 2-3 minutes at 37^{o}C incubator with 5% CO_2.
6. Add 7ml of DMEM to stop trypsin action.
7. Transfer all the mixture to a 15ml falcon tube to spin at 1g for 5 minutes.
8. Remove supernatant and resuspend with 5ml of DMEM media
9. Add 10ml of fresh DMEM media into petri dish and then add cell culture onto it.
10. Swirl the cell mixture to spread it evenly.

Seed mammalian cell into 12-wells plates

1. Repeat the same step(step2-8) as mentioned in passaging mammalian cell method.
2. For cell counting using hemocytometer, add 10ul of cells mixture into 40ul of trypan blue.
3. Mix well and add 10ul of mixture into each of the well of hemocytometer.
4. Count cells under microscope with 10x magnification. Blue-stained cells are not counted since they are dead
5. Seed 0.2-0.4 x 10^6 cells into each of the 12 wells plate.

6. Seed mammalian cells for 24 hours before proceed for transfection.

Transfection of mammalian cell (HEK293FT/HEK293)

1. Pre-warm OPTIMEM media (serum free media)
2. Pre-mix Turbofect(ThermoFisher) transfection reagent, DNA and OPTIMEM media.
3. For turbofect, 2ul is added for 1ug of DNA. Add 200ul of Optimem media into mixture.
4. Incubate the mixture for 30 minutes at room temperature inside the biosafety hood.
5. Remove media from each of the wells of 12-well plates. Add about 203-205ul of DNA mixture into each of the well.
6. Prepare for FACS analysis after 24 hours incubation at 37°C incubator with 5% CO_2.

FACS preparation

1. Pre-warm DMEM media, trypsin and 2%FBS with PBS buffer solution.
2. Remove media from wells.
3. Add 200ul of trypsin into each well of 12-well plate.
4. Incubate for 3-5 minutes at 37°C incubator with 5% CO_2.
5. Add 500ul of DMEM media into each well to stop trypsin reaction.
6. Transfer the cell mixture into Eppendorf tube and spin at 1g for 5 minutes to harvest cells.
7. Remove supernatant and avoid disturb the pellet. The mammalian cell pellet appears white color.
8. Resuspend cell pellet with 200-300ul of 2%FBS with PBS buffer solution.
9. Transfer cell mixture into cell strainer to ensure cells can be analysed with single cell profile.
10. Put the prepared cell strainer with cells inside on ice and use for later analysis.

Directed evolution protocol

1. Generation of libraries of mutants

(1) EZclone mutagenesis to generate megaprimers

10X mutazyme II reaction buffer	5ul
Template (#65767)	800ng
MY_2F	1.25ul
MY_2R	1.25ul
40mm dNTP mix	1ul
ddH2O	Top up to 50ul
Mutazyme II DNA Polymerase	1ul
Total	50ul

(2) EZcone reaction

Megaprimers	500ng
EZclone enzyme mix	25ul
Template	50ng
EZclone solution	3ul
ddH2O	Top up to 50ul
Total	50ul

(3) Digestion of amplification product from EZclone reaction

DpnI enzyme	1ul

Incubate at 37°C for 2hrs

- Transformation into Top10 competent cells

Use 100ul of competent cells, add 6ul of DpnI digested DNA into the competent cells and use heat shock transformation. Recover in LB.

- Plate onto chloramphenicol (Cml) plates and incubate at 37℃ for overnight.

2. Competition Assay

(4) Competition assay protocol

Electrocompetent cells	100ul
EZ1A (EZ4B)	50ng
1A:M13F (4B:M13F)	50ng

Use electroporation method, and recover in LB + Ampicillin(Amp). After 1hr, plate onto Cml+IPTG+Arabinose (CIA) plates. This completes the first round of competition assay.

3. Gibson assembly to recreate the selected mutant

(5) Q5 HIFI

NEB buffer	10ul
GC Enhancer	10ul
dNTP (10uM)	1ul
Primer_F (10uM)	1ul
Primer_R (10uM)	1ul
Template	1ul
Polymerase	1ul
ddH2O	Top up to 50ul
Total	50ul

Gel electrophoresis was conducted, and identified band was cut out and purified using gel extraction. We obtained two fragments of the plasmid.

(6) Gibson Assembly

The two fragments were assembled using ligation

Fragment 1	Total 5ul
Fragment 2	
2X HIFI DNA assembly master mix	5ul
Total	10ul

After incubation at 50℃ for 15min, Top10 competent cells were transformed with the ligated product using heat shock transformation. Then the cells were plated onto Amp plates.

4. QuikChange mutagenesis (Agilent Scientific) to recreate the selected mutants

(7) Mutant strand synthesis reaction

10X reaction buffer	2.5ul
DNA template	1ul
Primer 1	1.25ul
Primer 2	1.25ul
dNTP mix	0.5ul
QuikSolution reagent	0.75ul
ddH2O	17.75ul
Total	25ul
QuikChange Lightening Enzyme	0.5ul

(8) Digestion of the amplification product

DpnI enzyme	1ul

Incubate at 37℃ for 30min.

(9) Transformation into Top10 competent cells

Top10 competent cells	100ul
DpnI-treated DNA	6ul

Use heat shock transformation to transform the DNA into the competent cells. After that, the cells were plated onto Cml plates (diluted and concentrated). The next day, single colonies were picked to grow overnight broth, following with miniprep of the broth and send for sequencing to check whether the mutation sites were correct.

5. Ligation of the mutants with gRNA

(10)Digestion using BplI enzyme

DNA	2000ng
Tango	5ul
SAM	1ul
BplI	2ul
ddH2O	Top up to 50ul
Total	50ul

PCR cleanup was done after the reaction.

(11) SAP

Cleanup DNA	20ul
rSAP	1ul
Cutsmart Buffer	2ul
ddH2O	7ul
Total	30

PCR cleanup was done after the reaction.

(12) Oligo duplex annealing

gRNA_F	1ul
gRNA_R	1ul
10XT4 DNA ligase buffer	2ul
T4 PNK	0.5ul
ddH2O	15.5ul
Total	20ul

(13) Ligation

Digested/SAP plasmid	2ul
Oligo duplex	2ul
10XT4 ligase buffer	1ul
T4 DNA ligase	0.5ul
ddH2O	3.5ul
Total	10ul

After incubating at room temperature for 2hrs, the ligated plasmid was transformed into Top10 competent cells using chemical transformation. After recovering for 1hr, the cells were plated onto Amp plates.

(14) Colony PCR for gRNA ligation

gRNA_F	0.5
M13R_R	0.5
2X master mix	10
ddH2O	9
Total	20

6. Testing in human cell line

(15) Transfection

OptiMEM	500ul initially added to the DNA, and another 500ul to top up at the end
Turbofect	2ul
SpCas9 variants	1ug

The DNA-Turbofect-OptiMEM mixture was incubated at room temperature for 30min.

(16) Cell sorting (FACS)

- Cells were trypsinized with 200ul trypsin and incubated at 37℃ for 3mins
- Add 500ul media and centrifuge for 5mins, 1000rpm
- Remove supernatant and resuspend the cell pellet in 400ul 2%FBS in PBS
- Use cell strainer to filter cells into single cell and keep the tubes on ice
- Use FACS to sort out transfected cells

(17) Genome extraction

- After FACS sorting, spin down to collect cell pellet
- Resuspend pellet with 30ul of Quick Extract solution and vortex briefly
- Incubate samples at 65℃ for 15mins. Vortex briefly
- Incubate samples at 98℃ for 5mins
- Samples are ready for PCR

(18) PCR

Q5 High QC buffer	10ul
Q5 High QC enhancer	10ul
dNTP	1ul
Primer_F (10uM)	5ul
Primer_R (10uM)	5ul
Template (genome extraction product)	7ul
MQ water	11
DNA Q5 hot start polymerase	1
Total (per reaction)	50ul

(19) T7E assay

- Perform gel extraction of the PCR product
- Anneal the PCR product with 300ng DNA and 2ul NEB Buffer 2 (total volume per reaction is 19ul)
- Add 0.5ul T7 Endonuclease enzyme per reaction for digestion after annealing
- Incubate for 1 hr at 37℃
- Run gel to see the results